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Secretion of plasminogen activator and its inhibitor by glomerular epithelial cells

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Secretion of plasminogen activator and its inhibitor by glomerular epithelial cells. The effects of thrombin, interleukin-1 (IL-1), tumor necrosis factor (TNF) and γ-interferon (γ-IFN) on the release of plasminogen activator (PA) and inhibitor (PAI) were studied using cultivated human glomerular epithelial cells (GECs). Species of PAs and PAI secreted from the GECs were urokinase-type PA (u-PA) and tissue-type PA (t-PA), while the major species was a single chain u-PA in the amount of 28.6 ± 2.34 ng/10^5 cells for 24 hours (N = 4, mean ± SD), and PAI-1. The addition of increased concentrations of thrombin (0.1 to 31.6 U/ml) into confluent cultures enhanced the GECs to release u-PA, t-PA and PAI-1 in a dose- and time-dependent manner. The incubation of the GECs with 10 U/ml thrombin resulted in about a fourfold increase in the concentration of u-PA, threefold in t-PA and twofold in PAI-1. All thrombin effects, however, were suppressed by the simultaneous addition of cycloheximide, indicating that the enhancing effects of thrombin were due to an increase in the production of PAs and PAI-1, via protein synthesis. These thrombin effects appeared to be dependent upon the enzymatically active site of thrombin because DFP-thrombin had no effect. In the conditioned medium which was under continuous thrombin stimulation for 24 hours, no u-PA activity was detectable, even after the plasmin treatment, because a single chain u-PA was degraded by the thrombin. The stimulation of cultured GECs with thrombin only for the first three hours in 24 hour cultivation showed an apparent increase in the antigenic amount of u-PA. IL-1 enhanced the release of t-PA and PAI-1, and TNF did that of u-PA and t-PA, while γ-IFN showed no significant effects. These findings indicate that the GECs participate in the regulation of extracapillary fibrinolysis in the glomerular microenvironment, as being modulated by thrombin and two cytokines, IL-1 and TNF.

Plasminogen activator (PA) is a highly specific serine protease that converts the proenzyme, plasminogen, into an active proteolytic enzyme, plasmin. The two types of PAs, urokinase-type PA (u-PA) and tissue-type PA (t-PA) can be distinguished both physiologically and immunologically, and are present in several tissues and cultured cells [1]. It has been demonstrated that some cultivated cells secrete u-PA, t-PA, or both [2–4]. It has been known that PAs play an important role in fibrinolysis, cell migration, hormone processing and ovulation [1, 6–11].

It has been discussed that intraglomerular coagulation and fibrinolytic processes participate in the development and progression of various renal diseases [12–19]. Glomerular damage with fibrin deposition is occasionally followed by mesangiolysis and crescent formation [20, 21]. However, the relevance of glomerular intrinsic cells in the regulation of physiological fibrinolysis still remains almost unknown. Angles-Cano et al [22] examined the identification and cellular localization of PAs in human glomeruli by immunofluorescent technique and reported that t-PA was located in the endothelial cells and u-PA in the glomerular epithelial cells (GECs). They suggested that u-PA secreted from GECs related to a pathophysiological role of extracapillary fibrinolysis.

Thrombin is not only the key enzyme in the coagulation system but also acts as an anti-thrombogenic agent via thrombomodulin and protein C-protein S system [23–26]. It also has a direct influence upon the endothelial cells in the activation of the fibrinolytic system [2]. While cytokines such as interleukin-1 (IL-1), tumor necrosis factor (TNF) and γ-interferon (γ-IFN) also regulate the diverse functions of endothelial cells, macrophages and other cells, including coagulation and fibrinolytic systems. IL-1 (-α and -β) and TNF induce procoagulant activity due to the syntheses of tissue factor, PA inhibitor-1 (PAI-1), and suppression of protein C pathway; γ-IFN causes the increase of PA release from macrophages [6, 27, 28].

The purpose of this paper is to characterize the species of fibrinolysis-related mediators secreted from the GECs in vitro and to investigate the modulators affecting these fibrinolytic functions.

Methods

u-PA of a molecular weight of 55,000 was a gift from Mochida Pharmaceutical Co. (Tokyo, Japan). Human t-PA was purified from the conditioned medium of a melanoma cell line (Bowes) [29]. Latent type of human PAI-1 was purified from a conditioned medium of human fibrosarcoma cell line (HT1080) by a procedure of Andreason et al [30] and HPLC gel filtration chromatography (TSK gel G3000SW, Toyo Soda, Tokyo, Japan). Antibodies against t-PA and u-PA were obtained from immunized rabbits [2, 31], and antiserum against PAI-1 was taken from an immunized duck. The IgG fraction of both the rabbits and the duck was purified using ammonium sulfate precipitation and a column of either protein A-Sepharose or Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) coupled with goat IgG against duck IgG, respectively. The immunological specificity of each anti-IgG was examined with immunoelectrophoretic and Western blotting techniques using the respective crude and purified antigen samples. Anti-PA IgGs also quenched specifically the respective enzymatic PA

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activity in a dose dependent manner, but without immunological cross reactivity. Mouse monoclonal IgG to PAI-1 or PAI-2 were from Monozyme (Lyngby, Denmark) and Biopool AB (Umea, Sweden), respectively, and goat polyclonal IgG to PAI-1 was from Biopool AB. These monoclonal and polyclonal anti-PAI-1 IgGs, including duck anti-IgG produced by us, were all raised by latent type of PAI-1 and showed much less binding activity to PAI-1·t-PA or u-PA complex than that to free PAI-1 with Western blotting analyses. Monospecific IgG against u-PA, t-PA or PAI-1 was prepared by passing rabbit and duck anti-IgGs through a column of Sepharose 4B coupled with purified u-PA, t-PA or PAI-1. The absorbed IgG was eluted with 0.1 M glycine-HCl, pH 2.5. Fifty microliters of 1.0 M Tris-HCl, pH 9.0 was added to each eluate (1 ml). The IgG fractions, dialyzed against 0.01 M carbonate buffer, pH 9.5, were conjugated with horseradish peroxidase (HRP) by the periodate method [32]. Bovine alpha-thrombin was purified by cationic ion exchange chromatography from commercially available thrombin (Mochida Pharm. Co., Tokyo, Japan). The final preparation had a specific activity of 3600 U/mg of protein.

Diisopropylfluorophosphate (DFP) treated thrombin was prepared by reacting thrombin with 10 mM DFP at pH 7.2 for one hour at 37°C, and then dialyzed against 0.01 M phosphate buffered saline, pH 7.4 (PBS). The plasminogen was prepared from human plasma by the affinity column chromatography of lysine-Sepharose (Pharmacia) [33]. HRP, DFP, hirudin, human plasmin, bovine serum albumin and cycloheximide were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA), aprotinin from Bayer Co. (Leverkusen, FRG), and IL-1 (human, cell-line derived IL-1) from Genzyme Inc. (Boston, Massachusetts, USA). Single chain u-PA and TNF were from Mochida Pharmaceutical Co. (Tokyo, Japan) and γ-IFN was from Takeda Pharmaceutical Co. (Tokyo, Japan).

Isolation of glomerular epithelial cells

Fresh human adult kidneys were obtained at the nephrectomy for the localized tumors. The tissue samples were processed under sterile conditions by a modification of the method of Striker, Killen and Farin [34]. Briefly, the cortical segments were minced in Waymouth's medium (GIBCO Laboratories, Grand Island, New York, USA) containing 100 U/ml of penicillin and 100 μg/ml streptomycin. Then the segments were pushed through a 60-mesh stainless steel screen with a pestle and rinsed by Waymouth's medium. The filtrate was poured through a 100-mesh stainless steel screen. Remaining glomeruli on the 100-mesh screen were separated from tubular fragments by sufficient repeated passages over a 100-mesh screen. The final preparation was more than 98% free from fragments of both tubular tissues and Bowman's capsules by estimating with phase contrast microscope (Olympus, Tokyo, Japan).

Isolated glomeruli were incubated with clostridium collagenase (CLSPA, 750 U/ml, Cooper Biomedical Co., Freehold, New Jersey, USA) and passed over a 325-mesh stainless steel screen. After centrifugation of the filtrate, the sediment was resuspended with Waymouth's medium containing 20% fetal calf serum (GIBCO Labs) and epidermal growth factor (EGF, 10 ng/ml, Collaborative Research Inc., Lexington, Massachusetts, USA), and implanted in culture dishes coated with fibronectin (5 μg/ml, Collaborative Research), and incubated in an atmosphere of humidified air containing 5% CO_2. After about one week, colonies of GECs were subcloned using 0.1% trypsin and 0.02% EDTA in stainless steel cylinders. Third passaged cells were used in all experiments.

Identification of GECs

Epithelial morphologies of isolated cells were examined by phase contrast microscopy, scanning electron microscopy (SEM), transmission electron microscopy (TEM) and immunohistochemical methods. The cells grown on coverslips were prepared for SEM by fixation with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), postfixed in 1% osmium tetroxide, dehydrated in alcohol series, immersed in isoamyl acetate, and critical-point dried with carbon dioxide (HCP-2, Hitachi, Tokyo, Japan). After sputter-coating with platinum palladium, the cells were examined with a SEM (JSM-35CF, JEOL, Tokyo, Japan). For TEM, cells grown on glass slides were fixed the same as for SEM, dehydrated in alcohol series, and embedded in Epon 812. Some embedded samples were vertically re-embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a TEM (1200 EX, JEOL). These cells were studied immunohistochemically using PHM-5 (mouse monoclonal anti-human GEC IgG, Australian Monoclonal Development Pty., Artarmon, Australia), which reacted with surface polyanion of GECs after periodate-lysine-paraformaldehyde fixation at 4°C for 15 minutes [35], and rabbit anti-human von Willberand factor IgG (Dakopatts A/S, Glostrup, Denmark) and rabbit anti-human myosin IgG (Immunotech S.A., Marseille, France) after aceton fixation at 4°C for five minutes. The immunostaining procedures were carried out using a Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, Vermont, USA) and the peroxidase reaction was developed with diaminobenzidine. Indirect immunofluorescent study for cytokeratin antigens, using mouse monoclonal anti-human cytokeratin IgG (Becton Dickinson Immunocytometry Systems, Mountain View, California, USA) against cytokeratins with Moll's catalog numbers 8, 18, and 19 [36], and FITC-rabbit anti-mouse IgG (Miles Scientific, Naperville, Illinois, USA), was performed after methanol fixation at −20°C for five minutes and then aceton at −20°C for five minutes. All specimens were observed with an epifluorescent microscope (Olympus, Tokyo, Japan). To test for fibroblast contamination, the cells were cultured in Eagle's minimum essential medium containing D-valine instead of L-valine (GIBCO Labs) and 20% dialyzed fetal calf serum with D-valine medium [37].

Stimulation of GECs

GECs grown to confluency were washed with PBS for 10 minutes two or three times, and incubated with both serum- and EGF-free Waymouth's medium (600 μl/dish) added with thrombin (0.316 to 31.6 U/ml), IL-1 (1 to 10 U/ml), TNF (1.6 to 1,000 U/ml) or γ-IFN (1 to 1,000 U/ml). Stimulating media containing thrombin and cytokines were, moreover, added with polymyxin B sulfate (5 μg/ml, Sigma Chemical Co.) to inhibit the effect of endotoxin. The conditioned media were harvested after 24 hours of incubation and centrifuged at 10,000 rpm for five minutes. The supernatants were stored at −70°C.

To examine the effect of early and transient exposure to thrombin on the release of u-PA from the GECs, the GECs were incubated with thrombin (3.16 U/ml) for only the first three hours during 24-hour cultivation. In the thrombin-free control,

the medium was exchanged in the same manner to a new serum free medium at three hours, as in the case of the thrombin-stimulated GECs. The supernatants were harvested at 4, 6, 12 and 24 hours, centrifuged and stored at –70°C.

Effects of DFP-thrombin and cycloheximide

DFP-thrombin (0.033 to 10.5 μg/ml), DFP-thrombin (1.05 μg/ml) with thrombin (1.0 U/ml) and cycloheximide (10 to 1,000 nM) with thrombin (3.16 U/ml) were also applied to the GECs in the same manner as described above.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and fibrin autography

According to Laemmli [38], SDS polyacrylamide slab gels were prepared using a separating gel of 10% acrylamide (Nakarai Chemicals, Kyoto, Japan) and a stacking gel of 4% acrylamide. The electrophoretic mobility of the samples was estimated by comparing with those of high and low molecular weight standards (BIO-RAD Laboratory, Richmond, Virginia, USA). Fibrin autography was performed according to Levin and Loskutoff [3]. To determine the immunological nature of the PAs, 7 μg/ml of rabbit anti-u-PA IgG or anti-t-PA IgG was added to the fibrin agarose gel.

Immunoblotting assay for u-PA, t-PA and PAI-1 antigens

The conditioned media obtained from the cultures with or without thrombin stimulation were harvested after 24 hours and concentrated by using 2% deoxycholic acid and 24% trichloroacetic acid. SDS-PAGE of the concentrated media with or without reduction by 2% mercaptoethanol, and an electrophoretic transfer of the protein to a nitrocellulose sheet were performed according to the method of Towbin, Staehelin and Gordon [39]. The blot was reacted with rabbit anti-u-PA or t-PA IgG, or goat anti-PAI-1 IgG (1 μg/ml). Immunoreactive proteins were stained by the Vectastain ABC kit. The peroxidase was developed with 4-chloro-1-naphthol.

Enzyme-linked immunosorbent assay (ELISA) for u-PA, t-PA and PAI-1 antigens

The assay system used was a modification of that of Bergsdorf, Nilsson and Wallén [40]. In brief, rabbit anti-u-PA or t-PA IgG, or mouse monoclonal anti-PAI-1 IgG (100 μl, 5 μg/ml) in 0.1 M $NaHCO_3$ was placed on a 96-well microtiter plate (Coster, Cambridge, Massachusetts, USA) and kept overnight at 4°C. After washing and blocking with 1% bovine serum albumin, several diluted samples or antigen standards of u-PA, t-PA and PAI-1 were added to each well and kept at 4°C overnight. HRP-conjugated monospecific rabbit IgG (100 μl) against u-PA (0.1 μg/ml) or t-PA (0.5 μg/ml), and duck anti-PAI-1 IgG (1 μg/ml) were put into each well and incubated for three hours at room temperature. Then, after washing the plates, 0.1 M citrate phosphate buffer, pH 5.0, (100 μl) containing 0.4 mg/ml o-phenylenediamine and 0.01% H_2O_2 was added and incubated for about two hours in a dark and wet chamber at 37°C. The absorbance was measured at 405 nm using a multiscan spectrophotometer (Corona Co.). All samples were examined in duplicate. Standard curves of u-PA, t-PA and PAI-1 were reproducible and linear in a dose-dependent manner in the range of 0.1 to 10 ng/ml, 0.03 to 1.0 ng/ml, and 0.7 to 25 ng/ml, respectively.

Fibrinolytic activity of u-PA using chromogenic assay

According to the modified method of Lijnen et al [41], amidolytic activities of u-PA in samples with or without the plasmin treatment were measured with an S-2444 (3 mM, Kabivitrum, Stockholm, Sweden) in 0.05 M Tris-HCl, pH 8.8, containing 0.1 M NaCl, 0.01% Triton X-100 and 0.01% NaN_3. Rabbit anti-t-PA IgG (500-fold amount of 50% inhibitory IgG concentration, which was 0.24 μg/ml for 500 mIU/ml t-PA activity) was added to selectively measure the u-PA activity in the samples. Hirudin (20 U/ml) was also added to inhibit the direct hydrolytic effect of thrombin on S-2444, as well as aprotinin (100 KIU/ml), to inhibit the residual plasmin activity. After incubation with S-2444 at 37°C for several hours, the absorbance at 405 nm was measured using a multiscan spectrophotometer (Corona Co.). Fibrinolytic activity of t-PA was measured according to the method of Wiman, Mellbring and Rånby [42] using S-2251 (0.3 mM, Kabivitrum, Stockholm, Sweden), 0.1 mg/ml plasminogen, 70 μg/ml soluble fibrin and 20 U/ml hirudin, and rabbit anti-u-PA IgG (500-fold amount of 50% inhibitory IgG concentration, which was 0.3 μg/ml for 1 IU/ml u-PA activity) was added to suppress u-PA activity in culture media.

To examine the direct effect of thrombin on single chain u-PA, single chain u-PA (200 μg/ml) was incubated with thrombin (0.1 to 10 U/ml) in PBS containing 0.02% Tween 80, for 5 to 60 minutes at 37°C, and then u-PA activity after plasmin treatment was spectrophotometrically measured with S-2444 as described above.

Results

Culture of GECs

We used three passaged GECs for all experiments, because the GECs which were passaged more than four times were prone to show degenerative changes such as intracytoplasmic vacuoles and cellular shrinkage. We used dishes coated with fibronectin for the first and second passages to increase the plating efficiency. Fibronectin coating, however, was not necessary at the third passage of the GECs, because both good plating efficiency and cell growth were well maintained by seeding a full number of GECs. The third passaged GECs showed a confluent monolayer growth exhibiting a cobblestone appearance (Fig. 1a). Immunohistochemically, their cytoplasm was stained in a granular pattern with PHM 5 (Fig. 1b). By indirect immunofluorescent technique using mouse monoclonal anti-cytokeratin IgG, a large amount of reactive products was demonstrated in the cytoplasm of these cells (Fig. 1c). Upon examining with SEM, abundant microvilli were found on the surface of these cells (Fig. 1d). By TEM, tight junctions were observed. Neither peripheral dense bodies nor Weidel-Palade bodies were seen in their cytoplasm. These cells could not be stained immunohistochemically with either anti-von Willberand factor antibody or anti-myosin antibody. These findings were almost the same as those reported previously [43]. Cell growth activity was easily maintained in a medium containing D-valine substituted for L-valine, indicating that these cells were not fibroblasts. The contamination of endothelial cells, mesangial cells and fibroblasts was very improbable.

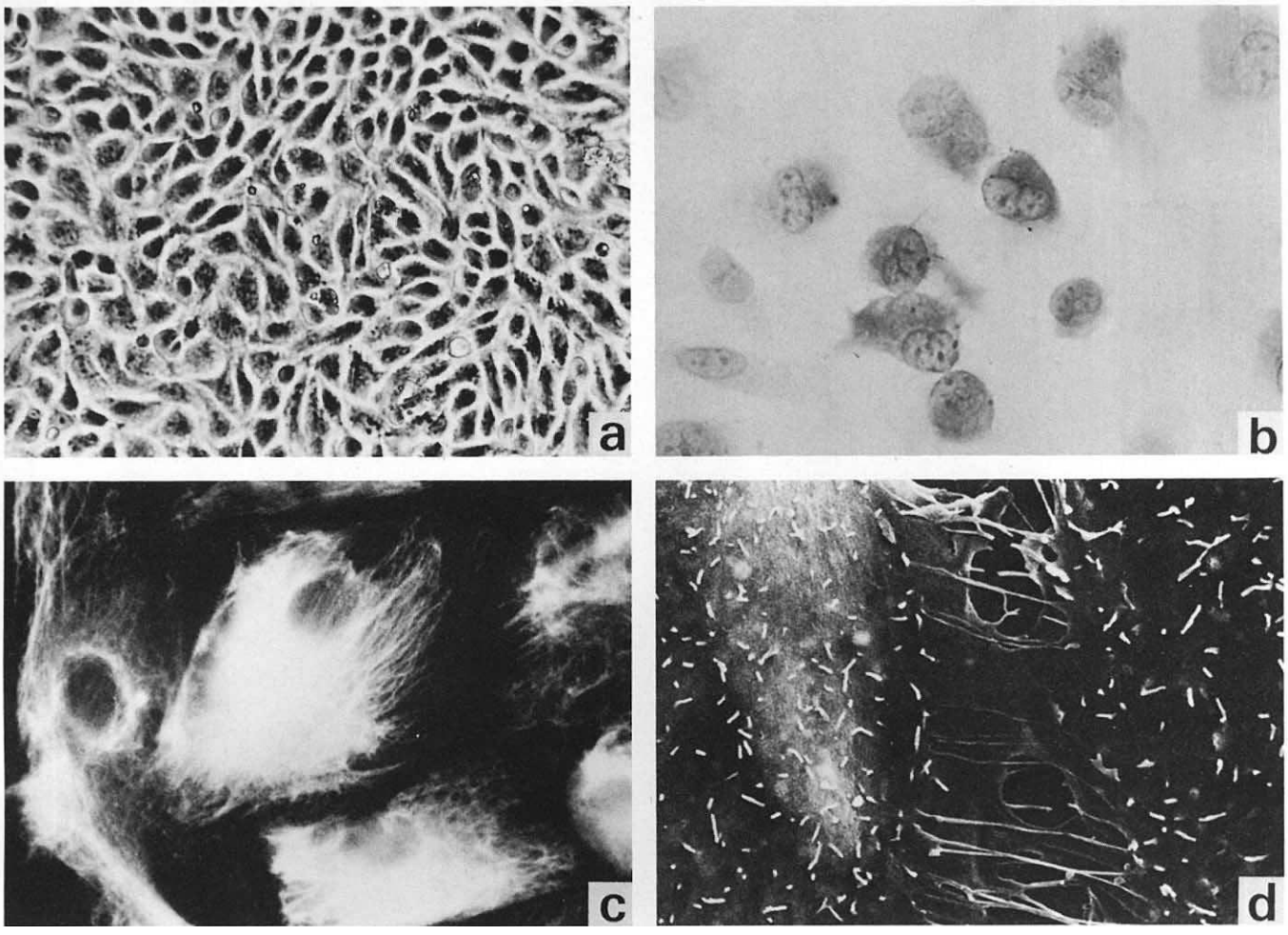

Fig. 1. a. *Cultured GECs at the third passage.* GECs show a cobblestone appearance. **b.** Immunohistochemical staining of GECs with PHM-5 antibody. **c.** Indirect immunofluorescence of GECs with mouse anti-human cytokeratin IgG. **d.** SEM of GECs. Many microvilli are present.

Characterization of PAs in conditioned medium of GECs

Fibrin autography revealed that GECs released PAs with molecular wts of 120,000 and 54,000 into the conditioned medium. The fibrinolytic activity of the band with a molecular wt of 120,000 was quenched by anti-t-PA IgG, and that with a molecular wt of 54,000 by anti-u-PA IgG (Fig. 2). Then, we assessed the effect of plasmin on the PA activities in the conditioned medium by fibrin autography and amidolytic activity. The fibrinolytic activity with a molecular wt of 54,000 was markedly enhanced after the plasmin treatment (Fig. 2), and this fibrinolytic activity was completely quenched by anti-u-PA IgG. The results of their amidolytic activities measured with S-2444 were similar to the enhancement of u-PA activity observed in fibrin autographies. Namely, the u-PA activity after the plasmin treatment was enhanced by about 100-fold, from 0.023 ± 0.001 to 2.54 ± 0.19 IU/10^5 cells. Immunoblotting analyses of the non-plasmin-treated conditioned medium under the presence of 2% mercaptoethanol, using anti-u-PA IgG, revealed a single band of a molecular wt of 54,000. However, the immunoblots of the conditioned media incubated with plasmin (0.001 CU/ml) at 37°C for one hour and reduced with 2% mercaptoethanol revealed a separation to two subunits of molecular wts of 33,000 and 21,000 (Fig. 3). These findings indicated that the majority of u-PA in the conditioned media was identical to single chain u-PA. Immunoblotting for t-PA revealed a single band with a molecular wt of 120,000. On the other hand, PAI-1 had a molecular wt of 50,000. After thrombin treatment, the PAI-1 related band changed to two bands with molecular wts of 50,000 and 47,000 (Fig. 3). PAI-2 related antigen was not detectable. In the conditioned media without any stimulant, the amount of u-PA antigen released from the GECs during 24 hour cultivation was about 28.6 ± 2.34 ng/10^5 cells; t-PA was about 0.58 ± 0.14 ng/10^5 cells, and the ratio of antigens between the released u-PA and t-PA was about 50.

Effect of thrombin on release of u-PA, t-PA and PAI-1 from GECs

The addition of increased concentrations of thrombin to the GEC cultures in serum free medium resulted in the increases in the levels of u-PA, t-PA and PAI-1 antigens in the conditioned medium in a dose dependent manner (Fig. 4). At a thrombin concentration of 3.16 U/ml, the levels of u-PA antigen almost reached a plateau. The incubation of GECs with 10 U/ml thrombin resulted in about a fourfold increase in the u-PA

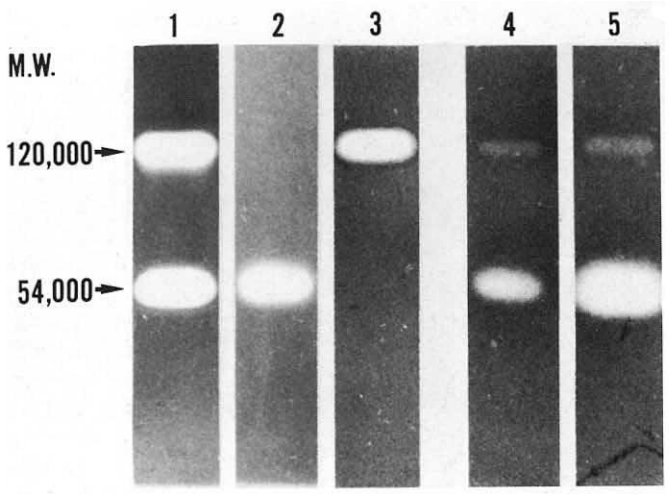

Fig. 2. *Fibrin autography of the conditioned medium of GECs after SDS-PAGE.* After 24-hr cultivation of GECs in the serum free medium, conditioned media were harvested. The conditioned medium contained PA activities with molecular wt of 120,000 and 54,000 (lane 1 and 4). The fibrinolytic activity with a molecular wt of 120,000 was quenched by anti-t-PA IgG (lane 2), and that with a molecular wt of 54,000 by anti-u-PA IgG (lane 3). The fibrinolytic activity of band with a molecular wt of 54,000 was markedly enhanced by plasmin treatment (0.001 CU/ml) for one hour at 37°C (lane 5) compared with that without plasmin treatment (lane 4). Lanes 1, 2 and 3 were incubated for 16 hr, and lane 4 and 5 for 6 hr at 37°C.

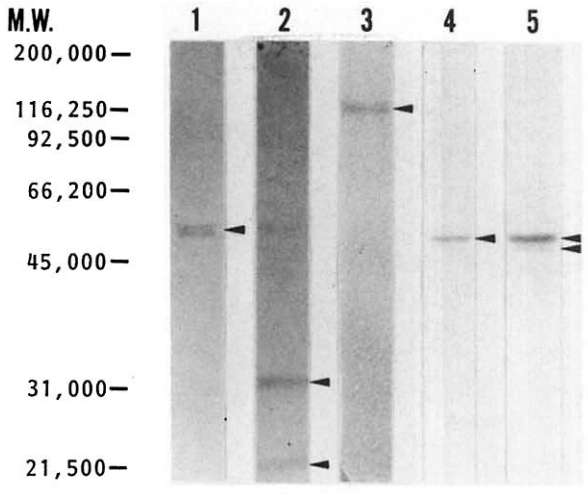

Fig. 3. *Western immunoblotting analyses for u-PA, t-PA and PAI-1 of conditioned medium.* After 24-hr cultivation in the serum free medium, conditioned media of the GECs were harvested. Lanes 1, 2 show immunoblots for u-PA, lane 3 for t-PA, lanes 4 and 5 for PAI-1. The lane 2 was treated with plasmin (0.001 CU/ml) for 1 hr at 37°C and reduced with 2% mercaptoethanol. The conditioned medium of the lane 5 was harvested after 24-hr cultivation under the thrombin stimulation (1.0 U/ml).

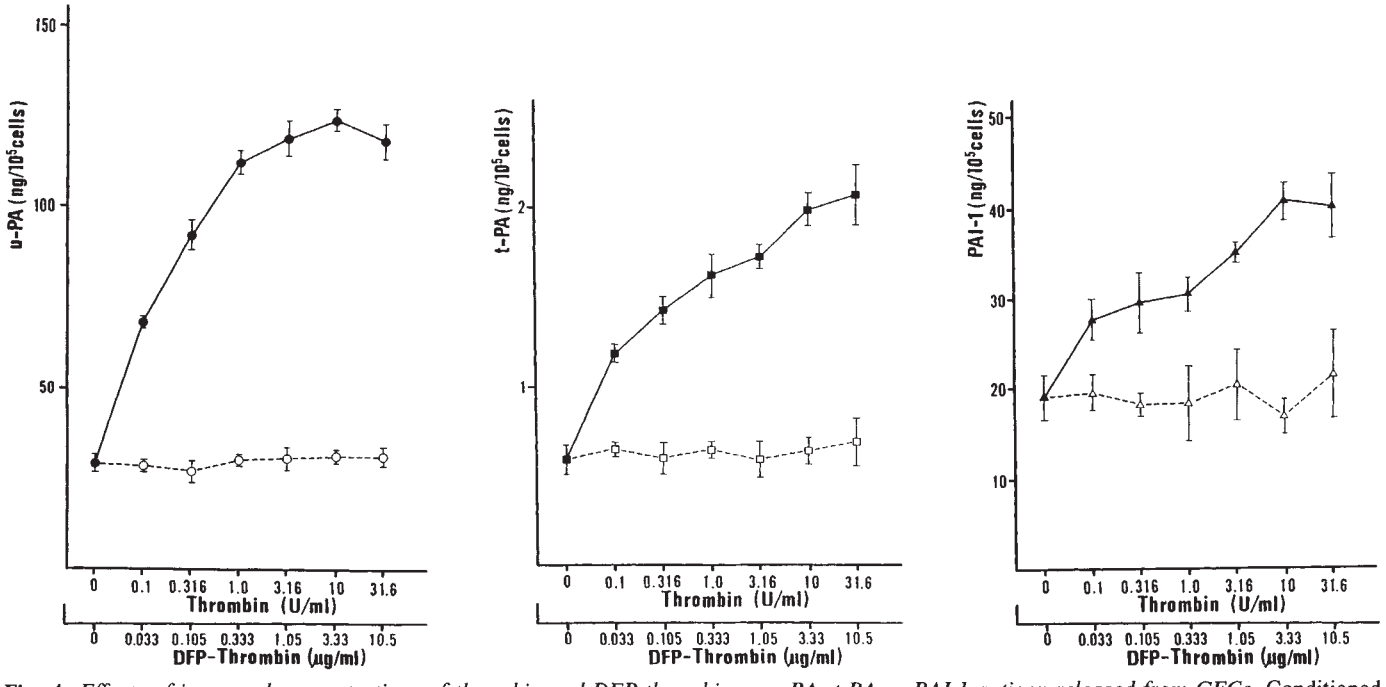

Fig. 4. *Effects of increased concentrations of thrombin and DFP-thrombin on u-PA, t-PA or PAI-1 antigen released from GECs.* Conditioned media were harvested after 24-hr cultivation. Thrombin stimulation showed a dose-dependent increase in antigenic levels of u-PA, t-PA and PAI-1. Symbols are: thrombin (●■▲), DFP-thrombin (○□△). Abbreviation is DFP, diisopropylfluorophosphate. $N = 4$; values are means ± SD.

concentration, threefold in t-PA and twofold in PAI-1. DFP-thrombin did not stimulate GECs to increase the release of the u-PA, t-PA and PAI-1 (Fig. 4). The level of u-PA, t-PA or PAI-1 antigen in the conditioned medium treated with both DFP-thrombin and thrombin was almost the same as that under the stimulation of thrombin only (Table 1). The time courses of u-PA, t-PA and PAI-1 antigens released from GECs are shown in Figure 5. The u-PA, t-PA and PAI-1 releases were increased

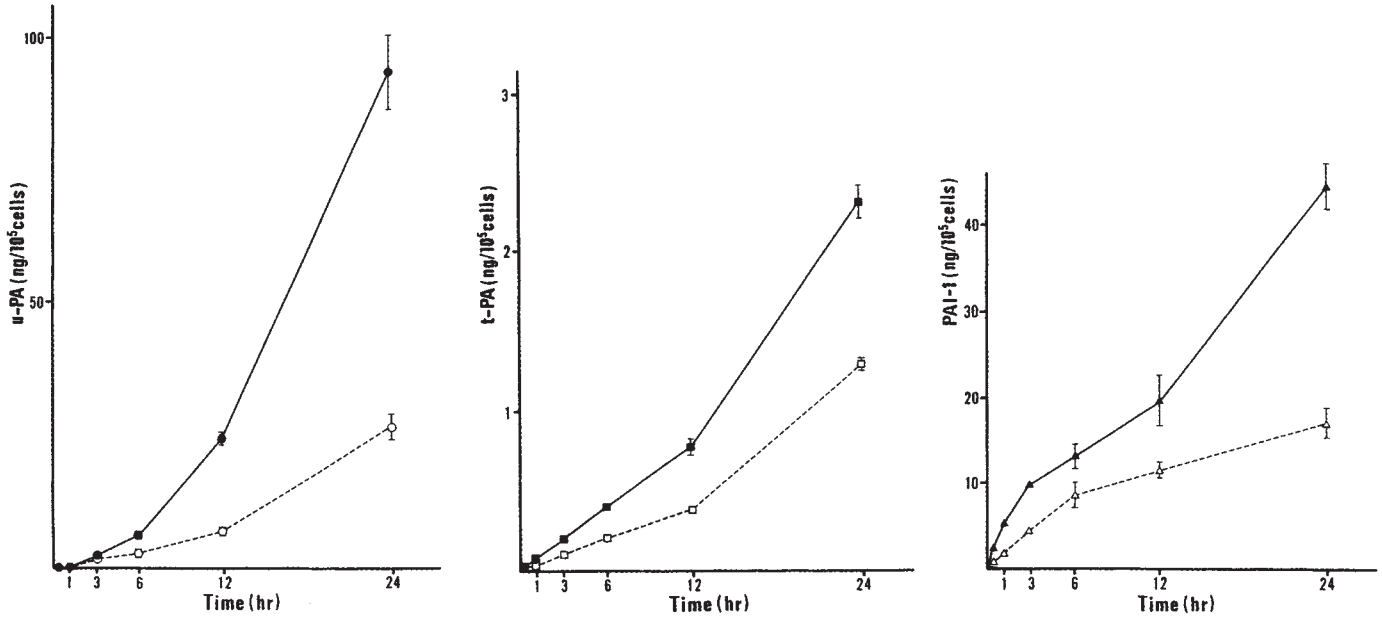

Fig. 5. *Time course of u-PA or t-PA or PAI-1 antigens released from GECs under thrombin stimulation (3.16 U/ml).* The releases of u-PA, t-PA and PAI-1 showed a time-dependent increase. Symbols are: thrombin (●■▲), control (○□△). $N = 4$; values are means ± SD.

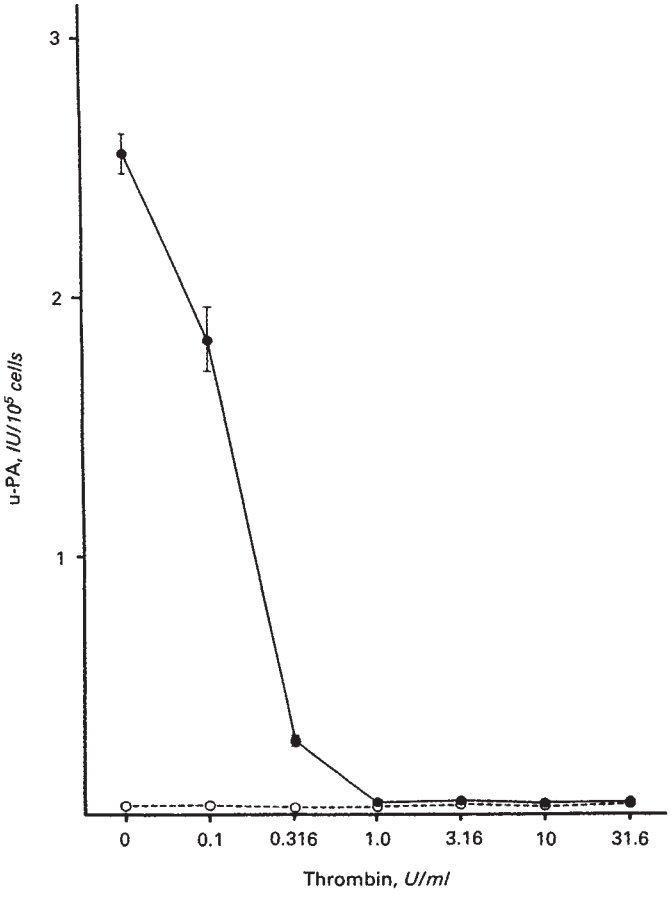

Fig. 6. *Effects of increasing concentrations of thrombin on u-PA activity in conditioned media.* Conditioned media were harvested after 24-hr cultivation under the thrombin stimulation and measured with (●) or without (○) plasmin (0.001 CU/ml) treatment for 1 hr at 37°C ($N = 4$, mean ± SD).

Table 1. Effects of DFP-thrombin on u-PA, t-PA and PAI-1 releases from cultured human glomerular epithelial cells

Treatment	u-PA	t-PA	PAI-1
	ng/10⁵cells		
None	29.5 ± 2.8	0.60 ± 0.06	18.7 ± 2.5
Thrombin *1.0 U/ml*	114.5 ± 3.4[a]	1.63 ± 0.10[a]	30.6 ± 1.9[a]
DFP-thrombin *1.05 μg/ml*	28.9 ± 2.7	0.60 ± 0.04	19.9 ± 3.8
DFP-thrombin *1.05 μg/ml* + thrombin *1.0 U/ml*	110.3 ± 8.9[a]	1.69 ± 0.11[a]	32.8 ± 4.3[a]

$N = 4$, values are means ± SD. Values were measured after 24-hour cultivation.

[a] $P < 0.01$ as compared to control values.

in a time dependent manner. In particular the u-PA release was parabolically increased after six hours, and the t-PA and PAI-1 after 12 hours.

In a continuous stimulation of GECs with thrombin, the u-PA activity in the conditioned medium after plasmin treatment was dramatically decreased (Fig. 6). The conditioned media, which was under thrombin stimulation for only the first three hours in 24 hour cultivation, showed an increase of u-PA antigen in a time dependent manner. But, these u-PA antigen levels were less than the continuously stimulated one with thrombin at respective incubation times. The u-PA activity after 24 hours was 4.8 ± 0.08 IU/ml only after plasmin treatment. However, no significant difference could be found between its activity level and the level for the control (Table 2).

Effect of cycloheximide

To examine whether thrombin enhanced the release of u-PA, t-PA and PAI-1 via the intracellular protein synthesis, cycloheximide (10 to 1,000 nM) was simultaneously added to the medium. Although the concentration of 10 nM had no effect, higher concentrations suppressed the levels of u-PA, t-PA and PAI-1 releases (Fig. 7). These findings revealed that thrombin

Table 2. u-PA antigen and activity under thrombin stimulation for only first three hour during 24 hour cultivation and continuous thrombin stimulation for 24 hours

	u-PA antigen *ng/ml*	u-PA activity *IU/ml*	
		Plasmin	
		non-treated	treated
Control	38.9 ± 0.5	ND	4.6 ± 0.2
Thrombin *3.16 U/ml*			
Only first 3 hr	68.0 ± 3.0[a]	ND	4.8 ± 0.1
Continuous 24 hr	180.1 ± 14.0[b]	ND	ND

GECs were stimulated with 3.16 U/ml of thrombin. All values were measured after 24-hour cultivation. Abbreviation is ND, not detectable (below 0.1 IU/ml). $N = 3$, values are presented as means ± SD.

[a] $P < 0.01$ as compared to control value

[b] $P < 0.01$ as compared to thrombin stimulated value for only first 3 hr

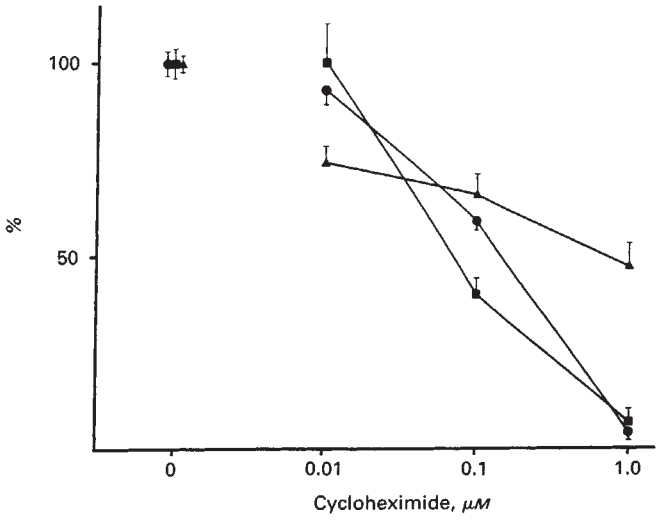

Fig. 7. *Effect of cycloheximide on the release of thrombin-induced u-PA, t-PA and PAI-1 antigens.* Simultaneous addition of cycloheximide decreased the release of u-PA (●), t-PA (■) or PAI-1 (▲) antigen from GECs under the 24-hr stimulation of thrombin (3.16 U/ml) in a dose-dependent manner ($N = 3$, mean ± SD).

enhanced the synthesis of u-PA, t-PA and PAI-1 antigens in GECs.

Effects of IL-1, TNF and γ-IFN on releases of PAs and PAI-1 from GECs

IL-1 stimulation (10 U/ml) resulted in more than a twofold increase in the t-PA concentrations in the medium, and about a 1.5-fold increase in PAI-1. On the other hand, IL-1 stimulation had no effect on u-PA release from GECs (Fig. 8A). TNF stimulation (200 U/ml), equivalent to 5.2×10^{-12} M, revealed 1.5 to twofold increases in u-PA and t-PA releases, and almost no effect on PAI-1 release. However, levels of them at 1,000 U/ml of TNF stimulation were conversely suppressed as compared with those at 200 U/ml of TNF, probably, suggesting the cytotoxicity of TNF because the shape of GECs changed to be spindle (Fig. 8B). γ-IFN had no stimulant effect on the releases of PAs and PAI-1 from GECs (Fig. 8C).

A change of u-PA activity under the stimulations of IL-1, TNF or γ-IFN after plasmin treatment was similar to that of u-PA antigen level, but no t-PA activities could be detected in them.

Direct effect of thrombin on single chain u-PA

As there was a discrepancy between the release of u-PA antigen and u-PA activity in the conditioned media treated with thrombin, the direct effect of thrombin on single chain u-PA was examined. When the purified single chain u-PA was incubated with thrombin, thrombin rapidly decreased the generation of u-PA activity in time and dose dependent manners (Fig. 9). But two chain u-PA activity was unaffected by the incubation of thrombin.

Discussion

In this paper, we demonstrated that cultured human GECs secreted u-PA, t-PA and PAI-1, and these syntheses and releases were modulated by thrombin, IL-1 and TNF, but not by γ-IFN.

The scavenger mechanism of extracapillary fibrin deposits in glomeruli is controversial in various glomerular diseases. The infiltrating macrophages and/or mesangial cells are considered to play a main role in the removal of fibrin deposits [44]. The fibrinolytic function of GECs has been also suggested to contribute to the extracapillary fibrinolysis in glomeruli by Angres-Cano et al from the immunohistochemical findings [22]. In the present study, PAs and PAI released from the GECs were characterized by quenching experiments and Western immunoblotting methods. u-PA was predominant in the PA species released by GECs. Particularly, most of the u-PA in the conditioned medium was a latent form of u-PA, namely single chain u-PA. This is compatible with the previous findings that single chain u-PA is the predominant extracellular form of PAs [7]. The secretion of u-PA by these GECs in serum-free conditioned media for 24 hours was about 29 ng/10^5 cells/0.3 ml and was much higher (approximately 14 times) than that by human foreskin microvascular endothelial cells [45], although there were some differences in culture conditions such as supplementation of 0.03% bovine serum albumin and passage numbers. On the other hand, t-PA existed mainly in the form with a molecular wt of 120,000, indicating t-PA·PAI complex as being judged from previous reports [2, 3].

Although epithelial cells of urinary tubules and transitional epithelial cells may represent a major source of u-PA in the urinary tract [1], we clearly demonstrated that cultivated GECs also synthesized and released single chain u-PA. The pathophysiological role of u-PA in the kidney still remains unclear. A part of the synthesized single chain u-PA binds to the cell-surface receptor for u-PA as cell-associated u-PA, other of them presents in the extracellular spaces either in a free form or complexed with PAI [46]. On the cell surface, plasminogen was converted to plasmin by the receptor-binding u-PA. This plasmin may not only play a fibrinolysis but also cleave directly the extracellular matrix including the basement membrane, and indirectly collagens due to the conversion of a pro-collagenase to active collagenase. Therefore, u-PA released from GECs may participate not only in extracapillary fibrinolysis but also in the regulation of matricial degradation in the glomeruli.

Endothelial cells from various sources were investigated minutely regarding the release of PAs in vitro. Many investiga-

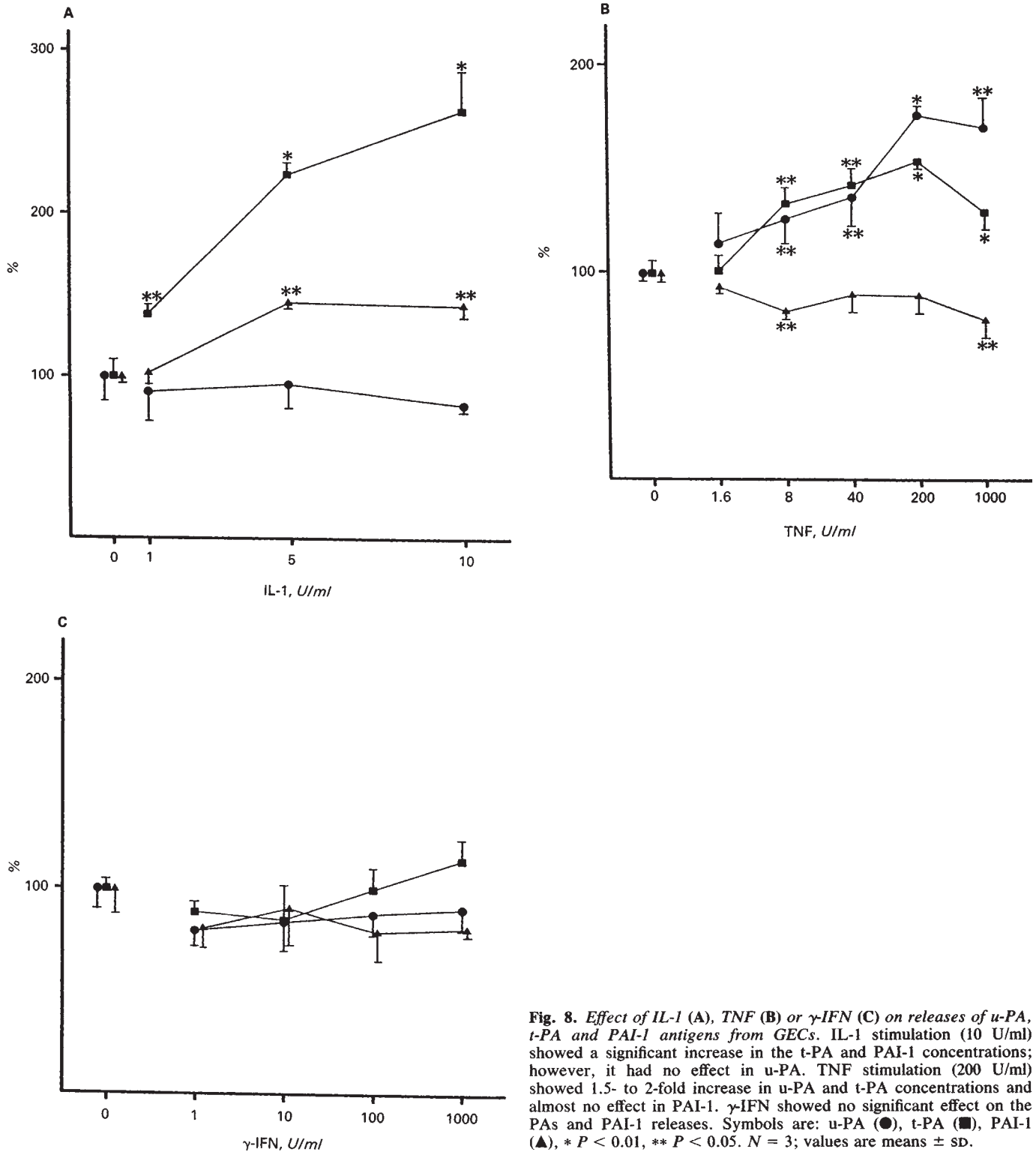

Fig. 8. *Effect of IL-1* (**A**), *TNF* (**B**) *or γ-IFN* (**C**) *on releases of u-PA, t-PA and PAI-1 antigens from GECs.* IL-1 stimulation (10 U/ml) showed a significant increase in the t-PA and PAI-1 concentrations; however, it had no effect in u-PA. TNF stimulation (200 U/ml) showed 1.5- to 2-fold increase in u-PA and t-PA concentrations and almost no effect in PAI-1. γ-IFN showed no significant effect on the PAs and PAI-1 releases. Symbols are: u-PA (●), t-PA (■), PAI-1 (▲), * $P < 0.01$, ** $P < 0.05$. $N = 3$; values are means ± SD.

tors reported that thrombin enhanced the secretion of PAs and/or PAI-1 from endothelial cells [45, 47, 48]. Nakashima, Sueishi and Tanaka also reported that thrombin stimulated cultured bovine venous endothelial cells to produce and release t-PA and PAI-1 in a conditioned media through its direct action on endothelial cells [2]. In the present study, we demonstrated that thrombin enhanced the release of u-PA, t-PA and PAI-1 antigens from the GECs into the serum free conditioned media, in dose- and time-dependent manners, and these effects were suppressed by the addition of cycloheximide. These findings

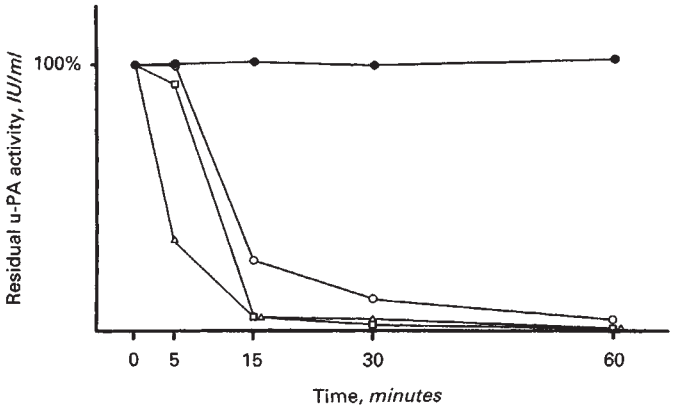

Fig. 9. *Inactivation of single chain u-PA with thrombin.* The activity of single chain u-PA (200 ng/ml) with thrombin treatment was rapidly decreased in time- and dose-dependent manners. A level of initial activity was expressed as 100%. u-PA activity was assayed with S-2444 as described in **Methods.** Symbols are: thrombin at 0 (●), 0.1 (○), 1.0 (□), and 10 (△) U/ml.

indicate that the enhancing effects of thrombin are mainly due to an increase in the production of u-PA, t-PA and PAI-1, via protein synthesis. Enzymatically inactivated thrombin with DFP did not increase PAs and PAI-1 levels in the conditioned medium, indicating that the enzymatically active site of thrombin was necessary to enhance the release of PAs and PAI-1 from GECs.

Loskutoff reported that u-PA activity in the conditioned medium decreased dramatically after the addition of thrombin in bovine aortic endothelial cells in vitro [47]. Thrombin has been shown to proteolytically cleave at Arg156-Phe157 of single chain u-PA to induce the inactive two chain u-PA [49, 50]. On the other hand, PAI-1 is also cleaved by thrombin, resulting in its conversion into inactive PAI-1 products [45, 51]. In our studies of the continuous stimulation of GECs with thrombin, u-PA activity could not be detected even after the plasmin treatment and the immunoblotting analyses for u-PA under the reducing condition revealed the two subunits of u-PA. These findings indicated that thrombin inactivated single chain u-PA and lost the ability of single chain u-PA to be converted into the active two chain u-PA. While, thrombin induces a release of PA from the vascular bed within a few minutes [52], and is rapidly inactivated mainly by binding with anti-thrombin III in plasma or on the endothelial surface [53]. Thrombin activity, however, is retained for many hours in the culture medium. Therefore, to investigate the enhancement of PA synthesis by thrombin from GECs, we performed the stimulation of GECs with thrombin for only the first three hours of a 24-hour cultivation instead of continuous stimulation for 24 hours. The level of u-PA antigen significantly increased after thrombin stimulation for the first three hours. This phenomenon indicated that the enhancing effect of thrombin on u-PA synthesis was present continuously after the removal of thrombin. On the other hand, the level of u-PA activity did not show a significant increase as compared with control one. The reason of this discrepancy between u-PA antigen and activity could not be resolved. Considering that our data obtained were analyzed in the fluid and fibrin-free condition, it is necessary to clarify the binding sites of thrombin and the modulation of fibrinolytic activity on a solid-phase such as fibrin or membrane surface of GECs.

Recent studies document that inflammatory cytokines are potent modulators of a variety of cell functions [27, 28, 54]. Infiltration of macrophages and lymphocytes has been shown glomerulonephritic diseases. The production of IL-1 and TNF by macrophages infiltrating in glomeruli was also reported to be stimulated by the deposition of immune complexes [55] and IL-1 is potent stimulant for the proliferation of mesangial and endothelial cells [56] and for renal prostaglandin synthesis [57]. Moreover, intrinsic cells such as mesangial and endothelial cells have also been shown to release IL-1 [58, 59]. As described above, IL-1 and TNF released from these cells have been suggested to offer various effects on glomerular intrinsic cells. Investigating the effects of IL-1, TNF and γ-IFN on PAs and PAI-1 releases from GECs, IL-1 and TNF have a stimulating effect upon PAs and PAI-1 releases from GECs, but γ-IFN does not, indicating that IL-1 and TNF produced in glomeruli may modulate the fibrinolytic function of the GECs. γ-IFN, however, may participate in a potentiation of other cytokines effects.

In conclusion, the facts that GECs secrete u-PA, t-PA and PAI-1, and thrombin, IL-1 and TNF modulate the fibrinolytic activity of GECs strengthen the hypotheses that GECs would participate in the regulation of extracapillary fibrinolysis in glomeruli and local inflammatory and immune processes could affect the fibrinolytic activity of GECs.

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